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Facilitative and competitive tradeoffs between *Morella cerifera* seedlings and coastal grasses

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science at Virginia Commonwealth University.

by

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Abstract

MORELLA CERIFERA SEEDLINGS MODIFY MICROCLIMATE AND VARY SEASONALLY FROM COMPETITIVE TO FACILITATIVE WITH NEIGHBORING GRASSES

By Michael Neumann Sinclair, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2019.

Major Director: Dr. Julie Zinnert, Assistant Professor, Department of Biology

Morella cerifera is a rapidly expanding native shrub on the Virginia barrier islands which displaces other native coastal species and may interrupt normal sediment dynamics. Barrier islands are considered stressful environments with low nutrients, high solar load, and frequent drought and salt exposure; facilitation often dominates in stressful environments according to the Stress Gradient Hypothesis. The objective of this project was to understand the importance of species interactions with grasses on the growth and physiology of *M. cerifera* at the seedling life stage through both field and lab experiments. Grasses provided ~1.3°C insulation to shrubs during winter freeze events and a freezing threshold for *M. cerifera* seedlings was experimentally found between -6°C and -11°C. Seedlings competed for light with grasses during warm months and grew more where grasses were clipped, revealing a tradeoff between winter insulation and summer light competition. *M. cerifera* shows evidence of ecosystem engineering at the seedling stage by significantly reducing summer maximum temperatures. This enables rapid expansion of *M. cerifera* across the landscape. As *M. cerifera* expands, island migration is altered, leading to decreased island stability and increased erosion. Although seedlings are small and relatively vulnerable, this life stage appears to have significant implications for the ecosystem trajectory and stability of the Virginia barrier islands.

Introduction

Until the last 30 years, ecological interaction research generally focused on competition and did not always take scale into account. There is a growing body of work supporting the importance of facilitation among species in understanding succession and ecosystem function (Maestre et al. 2009, Castanho et al. 2015). Facilitative effects are important to understand because, in the face of global climate change, abiotic-mediated change in distribution of species may be moderated by facilitation (Devaney et al. 2017).

According to the Stress Gradient Hypothesis (SGH), facilitation among species occurs in high stress environments, while competition dominates interactions among species at lesser stress levels (Bertness and Callaway 1994). Foundation species in harsh environments create favorable conditions for other species' seedling establishment by ameliorating temperature extremes and water loss. While it can be argued that these same foundational species are also competing with the plants under their canopy for light and belowground resources, stressful environments often allow for a net-positive interaction by moderating environmental stressors and alleviating nutrient limitations. This is often not the case in less stressful situations as greater resource availability favors competitive interactions (Angelini et al. 2011). The switch between dominance of competition and facilitation often occurs over very small spatial scales (Al-Namazi et al. 2017). While progress has been made on understanding how ecological interactions (and feedbacks among them) vary across scales, it is necessary to understand their effect on ecological processes and outcomes (Stachowics 2001, Stultz et al. 2006).

Facilitation is often most important during the life stages when an organism is most vulnerable, such as when the plant is a young seedling. During this stage, individuals need protection from harsh environmental conditions like excess solar radiation, heat, wind, and drought (Bruno 2000). In many grassland systems, grass canopies shade seedlings and ameliorate environmental stressors. Mature

grasses not only facilitate seedlings of their own species but often facilitate seedlings of other species, including shrubs (Maestre et al. 2001, Maestre et al. 2003). In this way, grasses can facilitate the establishment of shrubs and trees, possibly increasing the rate of succession. Facilitation can have large impacts on the successional trajectory of a system, even leading to the development of novel ecosystems (Walker et al. 2003, Angelini et al. 2011, Stinca et al. 2015). The interplay of facilitation and succession has been especially well-documented in the context of shrub expansion.

Global expansion of shrubs into grasslands has led to large changes in ecosystem diversity and function (Archer et al. 1995, Hiltbrunner et al. 2014). While facilitation by grasses and other species play a significant role, other factors are also important including increased winter temperatures, increased CO₂ levels, and changes in water availability (Archer et al. 1995, Morgan et al. 2007, Devaney et al. 2017, Huang et al. 2018). Shrubs that associate with nitrogen-fixing bacteria are particularly successful in nutrient-poor ecosystems (Hiltbrunner et al. 2014). The formation of shrub thickets with nitrogen-fixing microbial associates can have long-term effects on soil structure, processes, and nutrient availability (Zhou et al. 2017). Globally, it has been difficult to restore systems that have suffered invasion by shrubs and their nitrogen-fixing microbes back to a natural state due to nutrient legacy effects (Grove et al. 2015, Broadbent et al. 2017). This suggests that even in systems with encroaching native shrubs (not exotic), a return to the original ecosystem state may be impossible to achieve. This has direct implications for vegetation shifts being observed in coastal grassland sites including our study site, the Virginia Coast Reserve (VCR). Plants on the VCR barrier islands experience many stresses including extreme temperatures, nutrient-poor soils, drought, salt spray, and overwash (Young et al. 2011). Because of these environmental stressors, the islands of the VCR are an excellent place to study ecological interactions in a stressful environment (Leatherman et al. 1979, Zinnert et al. 2017).

Island vegetation change has been rapid, with a 40% increase of woody cover in the VCR observed in 27 years, predominately by one shrub species (Zinnert et al. 2017), *Morella cerifera* (wax

myrtle). This native, evergreen shrub is common on the barrier islands and the coastal plains of the southeastern United States. At the VCR, the salt-intolerant *M. cerifera* inhabits dune swales where it is protected from salt spray and is close to the freshwater lens (Zinnert et al. 2017). Due to its association with nitrogen-fixing actinobacteria, *Frankia*, (Wijnholds and Young 2000) and its ability to modify microclimate at the adult stage (Thompson et al. 2017, Huang et al. 2018), *M. cerifera* is considered a foundation species. It is dispersed mainly by birds and rapidly expands: within 15 years, a grass-dominated swale can become a closed-canopy shrub thicket (Zinnert et al. 2017). Few plants exist in the understory of mature thickets, but on the edges, survival and growth of species may be facilitated by *M. cerifera* (Thompson et al. 2017). Its northern congener, *M. pennsylvanica* has been shown to facilitate near-growing plants (Shumway 2000).

Previous work suggests that much of *M. cerifera*'s recent expansion on Hog Island is due to a warming of winter minimum temperatures (Figure 1). Grasslands rapidly convert to shrub-dominated thickets when minimum temperatures warm above a threshold of -15°C (Huang et al. 2018). Historically, these cold winter temperatures maintained grasslands as shrubs could not survive locations exposed to extreme temperatures. Due to climate change, winter minimum temperatures colder than this threshold have occurred only four times since 1985 (Huang et al. 2018). Previous work on *M. cerifera* freezing tolerance has focused on adult shrubs, leaving a gap in our knowledge of how seedlings, the leading edge of encroachment, respond to freezing temperatures.

Research on *M. cerifera* shrub expansion over the last decade has primarily focused on shrub physiology at adult life stages and interactions with abiotic stressors. The objective of this project was to understand the importance of species interactions with grasses on the growth and physiology of *M. cerifera* at the seedling life stage. A field experiment was conducted manipulating grass density (clipped vs unclipped plots) surrounding shrub seedlings, and a lab experiment was performed to determine shrub seedling freezing tolerance. It was hypothesized that grasses facilitate shrub growth through

microclimate moderation. I further hypothesize that *M. cerifera* seedlings will be less cold tolerant than adults and will have a warmer threshold (i.e. $> -15^{\circ}\text{C}$). By investigating these interactions and freezing effects, a better understanding of plant community dynamics can be attained as swale grasslands are invaded by *M. cerifera* in a changing climate.

Specific Objectives and Testable Predictions

1. Quantify *M. cerifera* seedling microclimate in the presence and absence of grasses

H: Ground temperatures and light levels will be moderated in plots where grasses are left unclipped in comparison to those with grass removed.

2. Measure performance and health of *M. cerifera* seedlings in the presence and absence of grasses

H: Juvenile *M. cerifera* shrubs under intact grass canopies will be healthier, have higher biomass, and have increased photosynthesis due to amelioration of extreme temperatures and excess solar radiation.

3. Quantify nitrogen mineralization in soils and total and fixed nitrogen in grass tissues in the presence and absence of shrub seedlings

H: Plots with *M. cerifera* seedlings will have similar nitrate and ammonium in soils and fixed nitrogen in grass tissues to plots without seedlings at this life stage.

4. Measure freezing tolerance of *M. cerifera* seedlings

H: Seedlings will exhibit a warmer freezing threshold than adult shrubs ($> -15^{\circ}\text{C}$).

Methods

Field Site: The Virginia Coast Reserve (VCR) contains 13 barrier islands on the Eastern Shore of Virginia, is owned by The Nature Conservancy, and is part of the Long-Term Ecological Research network (Hayden et al. 1991). The study site was the southern portion of Hog Island (37.417 N, 75.686 W), a barrier island. Shrub thickets first established in swales on the north end of the island and have been progressing rapidly southward (Young et al. 2007, Zinnert et al. 2011). This research focused on the leading edge of this invasion on the southern end of the island. The study location was dominated by coastal grassland species including *Ammophila breviguilata*, *Sporobolus pumilus*, *Solidago sempervirens*, and *Andropogon* spp. with many *M. cerifera* seedlings.

Experimental Design

In March 2018, 1 x 1m plots were established with three treatments (n=10 in each treatment): a young *M. cerifera* seedling and intact grass canopy (S), a *M. cerifera* seedling with grass clipped from the plot (SC), and intact grass canopy with all *M. cerifera* seedlings removed (G). All points were randomly chosen while controlling for environmental heterogeneity (Figure 2). Following initial clipping in March, resprouting grasses in SC were treated with glyphosate (KnockOut, 1.92%; Globe Chemical Company, Avondale Estates, GA) to eliminate belowground competition. Any further regrowth was reclipped as needed. To stop recolonization by grasses outside the plot, grass roots were severed at the plot edge using a shovel. Two SC plots were abandoned following complete mammal herbivory of the shrub seedlings in March and April. Replacement SC plots were established near the damaged plots in May. No further herbivory occurred.

Field Experiment

Microclimate and Shrub Measurements

DS1921 iButton data-loggers (Thermochron, Baulkham Hills, NSW, Australia) were installed in all plots to measure temperature 5 cm above the ground every 2 h from February 18 to August 7, 2018. Daily and monthly minimum and maximum temperatures were calculated from these temperature data. The 10% coldest and hottest temperatures of all records were also calculated. Daily high and low temperatures were obtained from the Painter, Virginia meteorological station from December 1955 until February 2019 for historical reference.

Light- adapted chlorophyll fluorescence (i.e. $\Delta F/F'm$) of the shrub seedlings (treatments S and SC) was measured in spring using a MINI-PAM-II photosynthesis yield analyzer (Walz Company, Effeltrich, Germany). Using a Li-COR 6400xt portable photosynthetic gas exchange system (Li-Cor, Inc. Lincoln, NE), photosynthetic rates were measured on one leaf per seedling in spring and summer. Ambient light conditions were matched and standard CO₂ conditions (400 ppm) used. Photosynthetically active radiation (PAR) was measured in each plot using a LI-1400 Datalogger (Li-Cor, Inc. Lincoln, NE) in spring and summer.

In each plot with a seedling (treatments S and SC), the height, maximum crown diameter, diameter perpendicular to the maximum diameter, and number of stems of the *M. cerifera* were measured in March, May, June, and August. Crown area was calculated by Equation 1.

Equation 1: Canopy Area = π * Maximum crown diameter * Perpendicular diameter

Change in height and canopy area was calculated by Equation 2.

Equation 2: Change= Final measurement – Initial measurement

Morella cerifera leaves were collected in summer, and leaf areas were measured using WinRhizo (Regent Instruments, Quebec, QC, Canada). Specific leaf area (SLA) was calculated by dividing leaf area by leaf dry mass. SLA positively covaries with photosynthetic rate and leaf N content. High SLA is associated with wider, thinner leaves; typically considered “cheaper” construction – low construction costs with high photosynthetic yield (Wright et al., 2005).

Grass Cover and Grass Tissue Analyses

To determine if nitrogen fixed by shrub seedlings was taken up by neighboring grasses and if grass water use efficiency differed in the presence of shrub seedlings, grasses were analyzed for carbon (C), nitrogen (N), $\delta^{15}\text{N}$, and $\delta^{13}\text{C}$. $\delta^{15}\text{N}$ shows the ratio of ^{15}N to ^{14}N ; negative $\delta^{15}\text{N}$ values are associated with nitrogen fixation. A more negative $\delta^{15}\text{N}$ would show that seedlings enrich the soil and grasses take those nutrients up. Grass samples were collected in the presence and absence of shrub seedlings (n=10). Samples were dried at 70°C for 72 hours, ground, and sent to the Cornell Stable Isotope Laboratory for analysis.

Soil Nitrogen Measurements

At all plots, ion-exchange resin beads (IERBs) were placed to measure biologically available nitrate and ammonium following the protocols developed by Nave et al. (2011). The IERBs were made of Dowex Marathon MR-3 mixed bed ion exchange resin beads. One IERB bag was deployed during the growing season at each plot in a 2.5 cm radius PVC pipe collar cut to 7.8 cm height. The collar was dug down into the soil, so that the top of the collar was level with the soil surface and then covered with removed soil. The IERBs were deployed for the growing season (May to September).

IERB bags and collars were collected and analyzed following Nave et al. (2011). IERB bags were rinsed with deionized water, and resins were removed from the bag. Resins were extracted in a specimen cup using 30 ml of 2M KCl overnight on a shaking table. The extractant was filtered through a

0.45 μm Durapore filter (3M Corporation, Minneapolis, MN) using a vacuum. The resulting solution was analyzed for NO_3^- and NH_4^+ content using a microplate reader to establish a mineralization rate (i.e. $\text{mg month}^{-1} \text{ cm}^{-3}$) following the protocol in Sims et al. (1995).

Storm Disturbance

In October, Hurricane Michael caused salt spray and minor overwash on the south end of Hog Island. The storm was followed by a lengthy low-pressure system which caused extensive salt spray and saltwater flooding. Following both events, shrubs were noticeably impacted. Recovery was estimated by the proportion of branches and branch tips with resprouting leaves and the proportion of pliable branches. Survival, number of green leaves, proportion of branch tips resprouting, and pliability of stems were recorded. There were several nor'easters in February and March 2019, possibly leading to more seedling salt exposure. In March 2019, survival, number of live leaves, and stem pliability were recorded again.

Freezing Experiment

Morella cerifera seeds were scarified and germinated in early September 2018 and transplanted to 15 cm pots in October, two per pot. All were grown in the VCU greenhouse for nine weeks. Seedlings were transplanted into nine cm pots, one per pot, and moved to an environmental chamber (Model #E15, Conviron, Winnipeg, MB, Canada) with 14 h of sunlight and kept at 25°C. After nine weeks, randomly chosen seedlings were moved to a walk-in refrigerator, kept at 4°C to mimic wintertime temperatures ($n=35$). Grow lights (GRO-LUX Wide Spectrum F-40, LEDVANCE, Wilmington, Massachusetts) were placed above the seedlings and LED construction lights to the sides (Craftsman 2000 Lumens LED Work Light, Craftsman, New Britain, CT; and Utilitech LED Construction Light, Utilitech, West Lawn, PA) delivering $\sim 400 \mu\text{mol m}^{-2} \text{ s}^{-1}$. A subset of seedlings remained in the 25°C environmental chamber to serve

as a warm control (n=7). Daily light duration was reduced to 10 h to simulate winter light conditions in January. Throughout the experiment, all seedling pots sat in a pan of water to reduce water stress.

Treatment Assignment and Prior Measurements

Seedlings were randomly assigned to freezing and control treatments. Freezing treatments were -20°C, -15°C, -11°C, and -6°C and control treatments were 4°C and 25°C (n=7). Prior to freezing, height was recorded with a ruler, general health was noted, and number of green, necrotic, and curled leaves recorded for each seedling. Dark-adapted chlorophyll fluorescence (i.e. Fv/Fm) was measured on the 4th fully expanded leaf using a MINI-PAM-II photosynthesis yield analyzer (Walz Company, Effeltrich, Germany).

Freezing and Recovery Measurements

To simulate an early morning winter freezing event, seedlings were placed in a chest freezer (Model FFFC09M1RW, Frigidaire, Charlotte, NC) that had been allowed to warm to 0°C. The target freezing temperature was achieved and then maintained for 180 min ($\pm 1^\circ\text{C}$) following Huang et al. 2018. After this time, the freezer was allowed to warm back to 0°C, and the seedlings were removed. Immediately following removal from the freezer, general health was noted, number of green, necrotic, and curled leaves was recorded, and dark-adapted Fv/Fm was measured. Leaf discs were excised from the youngest fully expanded leaves to measure electrolyte leakage described below.

One day and one week after freezing, survival and general health were noted, number of green, necrotic, and curled leaves was recorded, and dark-adapted Fv/Fm was measured for each seedling. After one week, seedlings were harvested, and aboveground fresh mass was recorded. Aboveground samples were dried at 70°C for 72 h and dry mass was recorded. Leaf discs from electrolyte leakage measurements were dried and added back to the biomass of plants from which they were removed.

Electrolyte Leakage Measurement

Thirteen leaf discs (1.27 cm or 0.5" diameter) were excised from the youngest fully expanded leaves following post-freeze measurements (n=3 due to destructive measurements). Fresh weight of leaf discs was recorded on a microscale, to estimate final biomass including removed leaf discs. The leaf discs were rinsed three times in DI water for two to three min each time (Jungklang et al. 2017). Leaf discs were placed into 15 ml of DI water in a 50 ml Thermo Scientific Nunc Conical Centrifuge Tube (Thermo Fisher Scientific, Waltham, MA) (Jungklang et al. 2017) and shaken at 50 rpm for 24 h (Boorse et al. 1998). Conductivity was measured using a conductivity probe (Oakton Benchtop Con 700 Meter with a K=1.0 probe, Oakton Instruments, Vernon Hills, IL). The tubes were autoclaved at 121°C for 15 min and shaken for 24 h at 50 rpm, after which maximum conductivity was measured. Relative conductivity was calculated by dividing initial conductivity by maximum conductivity (Boorse et al. 1998).

Statistical Analyses

All data were checked for normality and homogeneity of variance. One-way analysis of variance (ANOVA) was used to test differences in $\Delta F/F'm$, final biomass, and IERB nitrate concentrations among treatments. Maximum monthly temperature, minimum monthly temperature, PAR, light attenuation, photosynthetic rate, shrub canopy area change, and shrub height change among treatment and month were analyzed using two-ways ANOVA. Welch's *t*-test was used to test differences in total shrub canopy area growth, total shrub height growth, and September grass tissue $\delta^{13}C$, $\delta^{15}N$, and %N. Parametric test results were compared post-hoc using a Tukey test. Wilcoxon rank sum test was used to test for differences in SLA between S and SC plots. Kruskal-Wallis was used to test for differences in 10% coldest and hottest temperature records, IERB ammonium concentration, grass tissue $\delta^{13}C$, $\delta^{15}N$, and %N, and

relative conductivity. Nonparametric test results were compared post-hoc using a Nemenyi test or Fisher's least significant difference (based on sample size). Mixed-effects models were used to analyze differences in Fv/Fm and number of necrotic, curled, green, and total leaves among freeze treatments and sampling time with plant ID as a random effect. Poisson distributions were used with mixed effects models with response variables of counts. Importance of freeze temperature in each analysis was tested using a Likelihood Ratio Test. Post-hoc comparisons were performed using estimated marginal means, a least squares mean method over a reference grid. All analyses were tested at the $\alpha=0.05$ level.

Results

Field Experiment

Microclimate Modification

There was no significant ($F=1.58$, $p=0.09$) interaction of month and treatment for monthly maximum temperature, but main effects differed (month: $F=361.90$, $p<0.05$; treatment: $F=38.79$, $p<0.05$). All treatments significantly differed from one another. Interestingly, both treatments with intact grass canopies (G and S) had higher maximum temperatures than plots where grasses were clipped (SC) (Figure 3). Monthly minimum temperatures showed an interaction of treatment and month ($F=2.83$, $p<0.05$) (Figure 4). In the coldest month, March, there was a significant treatment effect ($F=10.69$, $p<0.05$) where SC plots had minimum temperatures that were 1.4°C and 1.2°C colder than G and S plots, respectively. The 10% hottest and coldest records differed significantly among treatments ($\chi^2=460.57$, $p<0.05$; $\chi^2=20.69$, $p<0.05$, respectively) (Figure 5). All treatments significantly differed from one another for the 10% warmest, but S and SC were not different in the 10% coldest records. The difference in 10% coldest temperatures may not be ecologically relevant as differences were <0.15 °C.

Light attenuation differed significantly for treatment ($F=6.72$, $p<0.05$) and month ($F=4.11$, $p<0.05$). There was no interaction ($F=0.67$, $p=0.52$). Light attenuation was less in clipped plots than plots with intact grass canopies and was $191 \mu\text{mol m}^{-2} \text{s}^{-1}$ greater in April than in August (Figure 6).

Seedling Physiology and Growth

In March, chlorophyll fluorescence ($\Delta F/F'm$) did not differ among treatments (S and SC) ($F=0.03$, $p=0.86$) (Table 1). Photosynthesis measurements significantly differed between June and August ($F=70.62$, $p<0.05$) but not between seedling treatments ($F=1.75$, $p=0.19$), and there was no interaction between month and treatment ($F=2.21$, $p=0.15$) (Table 1). SLA differed between treatments ($W=14$, $p<0.05$) SLA was higher in SC plots (Table 1).

Total growth did not differ between treatments (S and SC) for height ($t=0.06$, $p=0.95$) nor for canopy area ($t=-1.66$, $p=0.13$) (Figure 7). When growth was split between sampling periods, height growth and canopy area growth of shrub seedlings both showed a significant interaction of treatment and time period ($F=15.68$, $p<0.05$; $F=7.72$, $p<0.05$, respectively). Height change differed between S and SC from June to August as SC plots grew taller more rapidly than did S plots but did not differ between treatments before June (Figure 8). From March until June, canopy area growth was very similar between S and SC, but SC seedlings grew significantly more than S seedlings from June to August (Figure 9).

Nutrient Analyses

Ammonium and nitrate mineralization rates did not differ among treatments ($X^2=0.28$, $p=0.87$; $F=1.98$, $p=0.16$, respectively). Although nitrate concentrations did not significantly differ, there was a trend toward more nitrate in plots with shrubs, especially SC plots (Figure 10). $\delta^{13}C$, $\delta^{15}N$, and %N values did not differ in grass tissues at 0, 35, and 70 cm from a shrub seedling ($X^2=1.35$, $p=0.51$; $X^2=0.74$, $p=0.69$; $X^2=0.69$, $p=0.71$, respectively) (Figure 11). $\delta^{13}C$, $\delta^{15}N$, and %N values in grass tissues also did not differ in the presence or absence of a shrub seedling ($t=-1.60$, $p=0.13$; $t=-0.27$, $p=0.79$; $t=-0.90$, $p=0.38$, respectively) (Figure 12).

Recovery from Salt Exposure

On October 25, 2018, all but four seedlings had suffered total foliage loss with grasses providing no protection from salt spray (Figure 13a). Six of the 22 seedlings were releafing (Figure 13b). On March 29, 2019, 1 seedling still had green leaves (Figure 13c), three had pliable alive stems, and most had no alive aboveground tissues (Figure 13d).

Freezing Experiment

Freeze temperature was an important predictor of photosystem function as estimated by F_v/F_m ($\chi^2=218.92$, $p<0.05$). A threshold appears between -6°C and -11°C , below which frozen leaves and stems do not recover (Figure 14). Controls and -6°C were able to maintain photosynthetic function following treatment. The colder freeze treatments (-11°C , -15°C , and -20°C) showed some delay in the effects of damage but by a week after all were severely photoinhibited ($F_v/F_m < 0.52$). Relative conductivity differed among freeze treatments ($\chi^2=15.74$, $p<0.05$). Relative conductivity was greater in colder freeze treatments (-11°C , -15°C , and -20°C), as freezing damage led to more electrolyte leakage. The threshold estimated by this method was also between -6°C and -11°C (Figure 15).

Freeze temperature had a significant effect on number of necrotic and curled leaves ($\chi^2=112.95$, $p<0.05$; $\chi^2=69.85$, $p<0.05$, respectively). There were very few necrotic leaves in any of the treatments until one week after freezing, and only in plants frozen to $\leq -11^\circ\text{C}$ (Figure 16). Number of curled leaves increased with colder freezes and with time after the freeze event; though all seedlings exposed to 4°C or colder had some curled leaves. Freeze treatment had a significant effect on number of green leaves and total leaves ($\chi^2=246.19$, $p<0.05$; $\chi^2=37.32$, $p=0.01$, respectively). The 25°C treatment had more green leaves and more leaves total at all time steps than other treatments. All treatments exposed to cold had fewer total leaves and lost very few leaves after freezing, as leaves became necrotic but did not

drop. Plants frozen to $\leq -6^{\circ}\text{C}$ had fewer green leaves a week after freezing (Figure 17). Final biomass did not differ among treatments ($F=1.53$, $p=0.25$).

Extreme Temperatures

From year to year, average winter minimum temperatures have increased (Figure 1). Temperature events colder than -11°C , a conservative estimate of the seedling freeze threshold, have occurred 44 times since 1985 at the Painter, Virginia meteorological station. Accounting for the 1.3°C insulation provided by grasses, only 20 $< -12.3^{\circ}\text{C}$ events have occurred (Figure 18). During the field experiment, only one temperature event occurred below -11°C (-12.8°C) in Painter, VA, but no plot iButtons showed minimum temperatures $< -7.5^{\circ}\text{C}$. Temperatures $> 50^{\circ}\text{C}$ occurred 13 times during the study but never in a shrub plot and mostly in one grass plot (Figure 19). *M. cerifera* can maintain photosynthesis up to 50°C (Young 1992).

Discussion

Understanding the mechanisms that control early stages of shrub encroachment is important for comprehension of whole-island sediment dynamics. As shrubs expand into barrier island grasslands, they interrupt normal island rollover and increase erosion, endangering the future stability of barrier islands (Zinnert et al. 2017, Zinnert et al. in press). Microclimate modification in adult shrub thickets has been demonstrated as a mechanism for the shift from grassland to shrubland; however, the role of the seedling stage has not been investigated. It was hypothesized that microclimate would be modified in plots with intact grass canopy and that this modification would cause enhanced physiology (e.g., photosynthesis) and growth of shrub seedlings. My hypothesis was partially supported as grasses modified microclimate through increased light attenuation and winter insulation from minimum temperatures; however, shrub seedlings exhibited higher growth in the summer where grasses were removed by clipping, revealing a net competitive interaction at this life stage. As hypothesized, plots with shrub seedlings did not have more fixed nitrogen in neighboring grasses or higher rates of nitrogen

mineralization, but there was a nonsignificant trend toward more nitrogen mineralization in plots with shrubs, suggesting that seedlings may enrich soils at later stages. The hypothesis that seedlings have a warmer freezing threshold than adults ($>-15^{\circ}\text{C}$) was supported as seedlings had a threshold between -6°C and -11°C , below which seedling physiology and electrolyte leakage measurements showed unrecoverable damage to aboveground tissues.

While grasses did ameliorate winter minimum temperatures and protected *M. cerifera* seedlings from freezing events, grasses did not moderate extreme summer temperatures. However, plots with shrub seedlings had lower maximum temperatures than those without, demonstrating that *M. cerifera* moderates microclimate at earlier life stages than previously found (Thompson et al. 2017, Wood et al. in prep). This is significant and demonstrates that *M. cerifera* is an ecosystem engineer at the seedling stage by creating environmental conditions more favorable for growth, possibly leading to faster expansion. Grasses did attenuate light, hypothesized to be beneficial in a high-light environment like barrier island swales, but this did not translate to increased seedling $\Delta F/F'm$ or photosynthesis (Table 1). Conversely, SC seedlings were as tall and had wider canopies than S seedlings (although not significantly), suggesting that shrub seedlings are competing with grasses for light, as has been seen in other systems (e.g. Davis et al. 1998) However, as shrubs are rarely found in bare soils, the presence of grasses is beneficial to shrub seedlings at some earlier point in growth (Baucom unpublished data).

There may be a tradeoff for shrub seedlings between summer light competition and winter facilitation. Shrub seedlings which grow in dense grasses experience increased competition for light but are less likely to suffer mortality in a freeze event. Temperature events colder than -11°C , a conservative estimate of the threshold found in the freezing experiment, have occurred 44 times since 1985, when shrubs began to rapidly expand across the landscape (Young et al. 2007, Zinnert et al. 2011). Seedlings are vulnerable during these relatively common events and would likely experience irreversible damage if not other for other protection, including insulation by grasses. Accounting for the 1.3°C insulation

provided by grasses, fewer $< -12.3^{\circ}\text{C}$ events have occurred (Figure 18). Insulation by grasses considerably reduced the frequency of potentially lethal temperatures for seedlings (D'Odorcio et al. 2012). The interaction between grasses and *M. cerifera* seedlings varies seasonally from facilitative to competitive.

This temporal shift between facilitation and competition has been demonstrated in other systems where facilitation dominates during stressful times and competition dominates during less stressful times (Maestre et al. 2003, Kikvidze et al. 2006, Wright et al. 2014, Loranger et al. 2017). Although we expected summer to be stressful for shrub seedlings, we found that grasses did not ameliorate maximum temperatures and shrub seedlings competed with grasses for light. It appears that summer temperatures may not be stressful for *M. cerifera* seedlings. *Morella cerifera* has a relatively high photosynthetic temperature optimum ($\sim 30^{\circ}\text{C}$) and maintains some level of photosynthesis (although reduced) up to 50°C (Young 1992); no temperatures occurred above 50°C in any shrub plots (Figure 19). Shrub response from the freezing experiment suggests that winter may be the most stressful time of the year for shrub seedlings. As winter minimum temperatures in the region have warmed due to climate change (Goldstein et al. 2018), winters have become less stressful for *M. cerifera* seedlings. Thus, interactions between shrub seedlings and grasses have likely become increasingly competitive, providing evidence that species interactions shift with climate change (Van der Putten et al. 2010, Klanderud 2005), maybe enhancing expansion rates across the landscape.

Grasses likely facilitate *M. cerifera* germination and very early stages of seedling growth (earlier than this experiment). As seed dispersal is not a limiting factor within 300 m of shrub thicket, patterns in seedling distribution are due to microclimate at the location of dispersal (Woods et al. in review). *M. cerifera* seedlings grow most frequently in intermediate grass densities and infrequently in bare sand (Baucom unpublished data). Very young *M. cerifera* appears to benefit from grass amelioration, but by

the time that seedlings grow to 15 cm tall (the average starting height in the field experiment), the interaction is net competitive to neutral.

Grasses appear to benefit little from interactions with shrub seedlings; *M. cerifera* competes with and excludes grasses (Young et al. 2007, Thompson et al. 2017, Zinnert et al. in press), but grasses may profit from increased soil nitrogen at later shrub stages. Although not significant, plots with shrub seedlings had greater nitrate concentrations than those without, suggesting a trend toward increased soil N as shrubs age. Changes in $\delta^{15}\text{N}$ and %N were not detected in leaves of grasses neighboring shrub seedlings, suggesting that they are not incorporating fixed nitrogen into aboveground tissues; however, root material was not sampled. While grasses may not benefit from *M. cerifera* nitrogen fixation at this life stage, previous work has shown that there is greater soil nitrogen under thickets (Brantley and Young 2008) and a trend toward more soil nitrogen near intermediate-aged, free standing shrubs compared to grassy areas (Thompson et. al. 2017). *Baccharis halimifolia* neighboring *M. cerifera* thickets incorporates fixed nitrogen provided by *M. cerifera* (Vick 2011); as *M. cerifera* seedlings grow larger, grasses may incorporate fixed nitrogen, while *M. cerifera* will have had more time to build up soil nitrogen by litter drop, as *M. cerifera* does not resorb most leaf nitrogen before senescence (Brantley and Young 2008). As litter breaks down, much of the nitrogen travels to groundwater where it is available for uptake by plants (Zinnert unpublished data). Although adult *M. cerifera* enriches soils, it rapidly excludes other species, outweighing the potential soil enrichment benefits and demonstrating a net competitive interaction with neighboring plants seen in other invasions by plants associated with nitrogen-fixing bacteria such as *Cytisus* (Haubensak and Parker 2004, Wearne and Morgan 2004).

Morella cerifera seedlings compete with grasses for light and grow more when grasses are clipped away. When grasses were dormant March to May, S seedlings had an average 58% increase in height - a competitive advantage for the evergreen *M. cerifera* over coastal grasses which grew very little in this time period (Figure 8). S seedlings had an increase of ~600% in canopy area during the study

(Figure 9); seedlings are shading out and excluding other species earlier than previously observed. At later stages, competitive effects intensify as shrub thickets exclude nearly all other species (Zinnert et al. 2011, Wood et al. in prep). Once the shrub canopy is closed, light values under the canopy and litter are extremely low, starving the seedbank of light and suppressing the survival or germination of any understory plants (Brantley and Young 2007). Thickets arrest succession under current climatic conditions, preventing the historic progression to maritime forest and creating ecological scenarios not experienced before in coastal systems (Bissett et al. 2016, Zinnert et al. 2017).

This new ecosystem is a result of ecosystem engineering by *M. cerifera*. Seedlings modify microclimate and enrich soils which, combined with warming winter temperatures, enables the rapid expansion of *M. cerifera* across the barrier islands. As *M. cerifera* expands, it may decrease the amount of sediment moved cross-island. Increased sediment stability on barrier islands may prevent rollover, a barrier island's normal response to sea level rise and cause increased island area loss (Zinnert et al. 2017, Zinnert et al. in press). Although *M. cerifera* seedlings are small, the biotic interactions which govern their success appear to have significant implications for successional trajectories and perhaps even whole-island stability (Walker et al. 2003, Angelini et al. 2011, Zinnert et al. in press). Few studies investigating shrub expansion look at the role of the seedling life stage even though shrub seedlings are the leading edge of encroachment. While much is known about how adult shrubs interact with nearby species (Shumway 2000, Al-Namazi et al. 2017), modify microclimate (Devaney et al. 2017, Thompson et al. 2017), and respond to disturbances and climate change (Archer et al. 1995, Morgan et al. 2007, Huang et al. 2018), these are understudied at younger life stages. Biotic and abiotic factors, which control seedling success, influence later vegetation and landscape patterns (Walker et al. 2003, Angelini et al. 2011, Stinca et al. 2015). Therefore, it is necessary to understand the dynamics of shrub encroachment at all life stages in order to better manage and study this global phenomenon.

Table 1. Mean (\pm SE) of photosynthesis in June and August, $\Delta F/F'm$ in April, and SLA in August. Letter codes signify significant differences. Capital letters represent significant differences for two-way ANOVA (treatment * month) and lowercase letters represent differences from one-way ANOVA (treatment).

	Spring		Summer	
	S	SC	S	SC
Photosynthesis	19.28 \pm 1.77 ^A	22.55 \pm 1.36 ^A	11.48 \pm 0.54 ^B	11.35 \pm 0.66 ^B
$\Delta F/F'm$	0.16 \pm 0.03 ^a	0.19 \pm 0.04 ^a	-	-
SLA	-	-	0.87 \pm 0.08 ^a	0.99 \pm 0.01 ^b

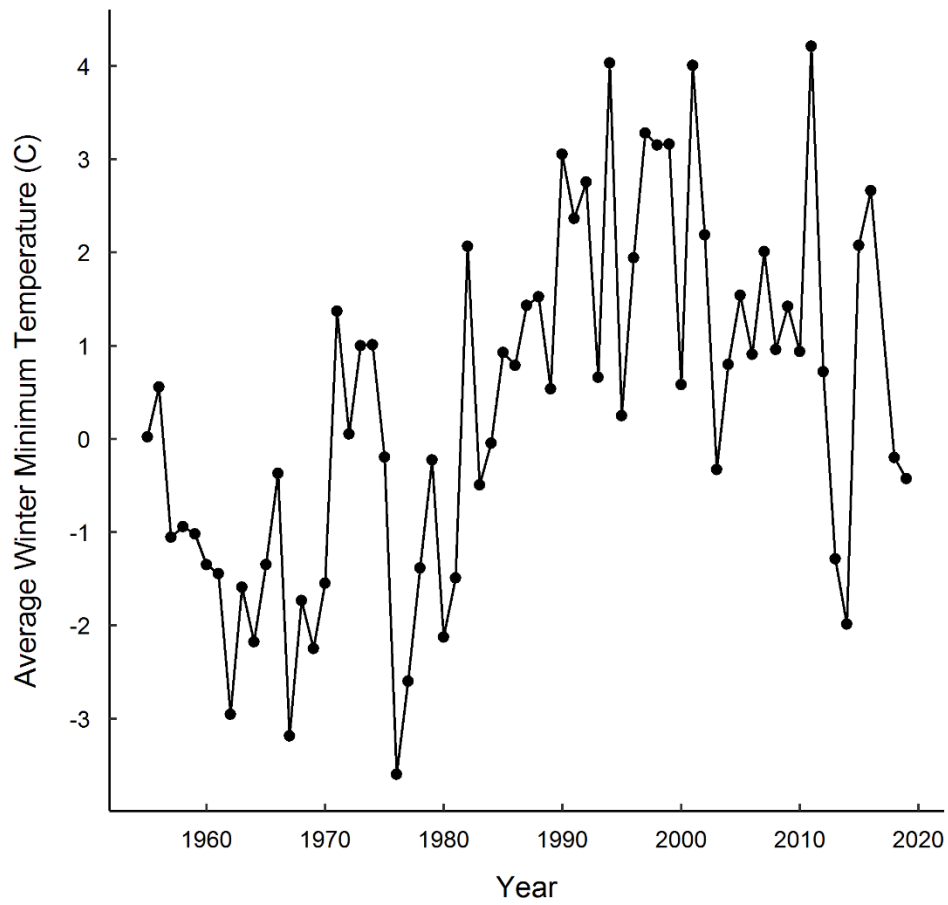


Figure 1. Line graph of average winter minimum temperature near the study site in Painter, Virginia from 1955 to 2019. Winter minimum temperatures have been increasing, likely contributing to the expansion of *M. cerifera*

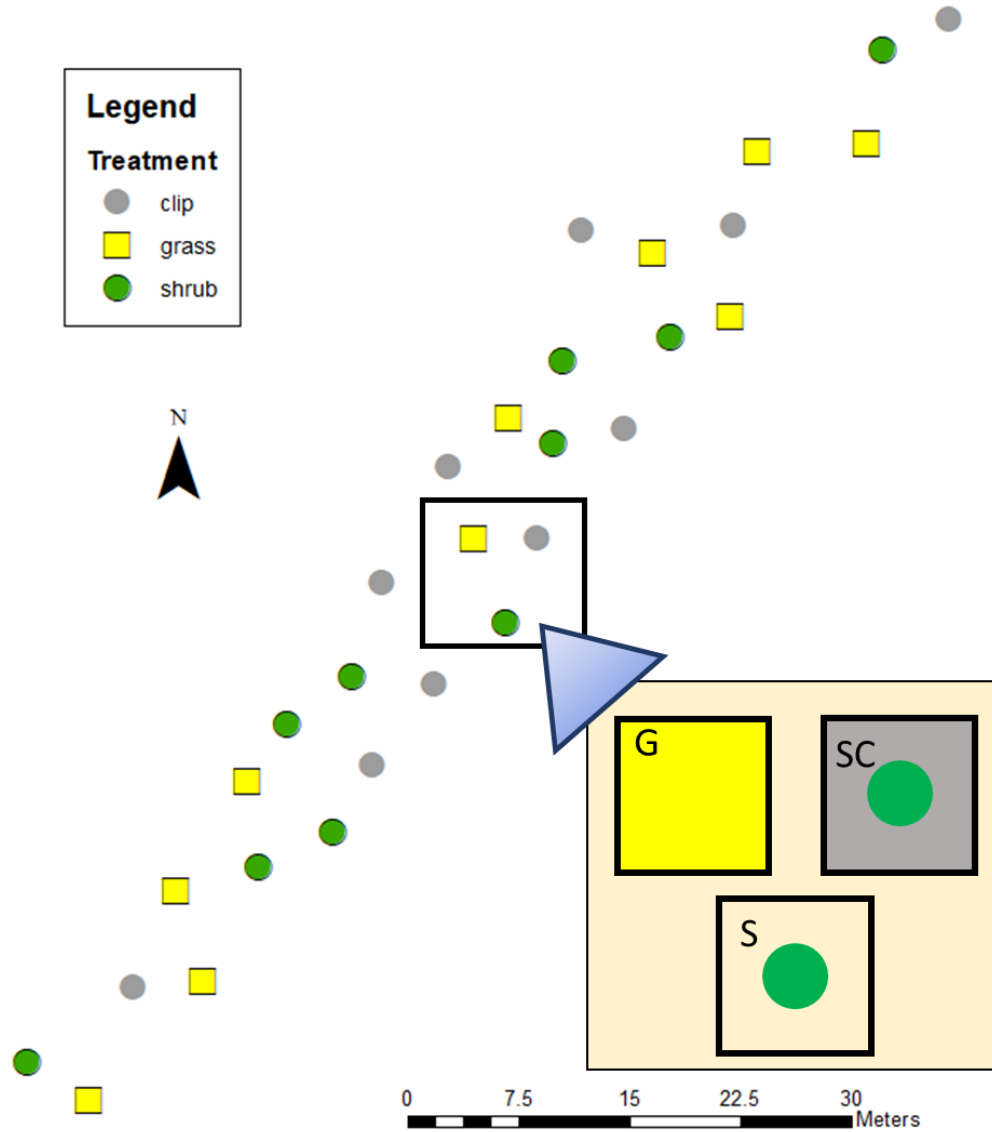


Figure 2. Experimental design with thirty 1x1 m plots in the study area. Inset shows three treatments: grasses only (G), *M. cerifera* seedling with grasses clipped (SC), and *M. cerifera* seedling with grasses intact (S).

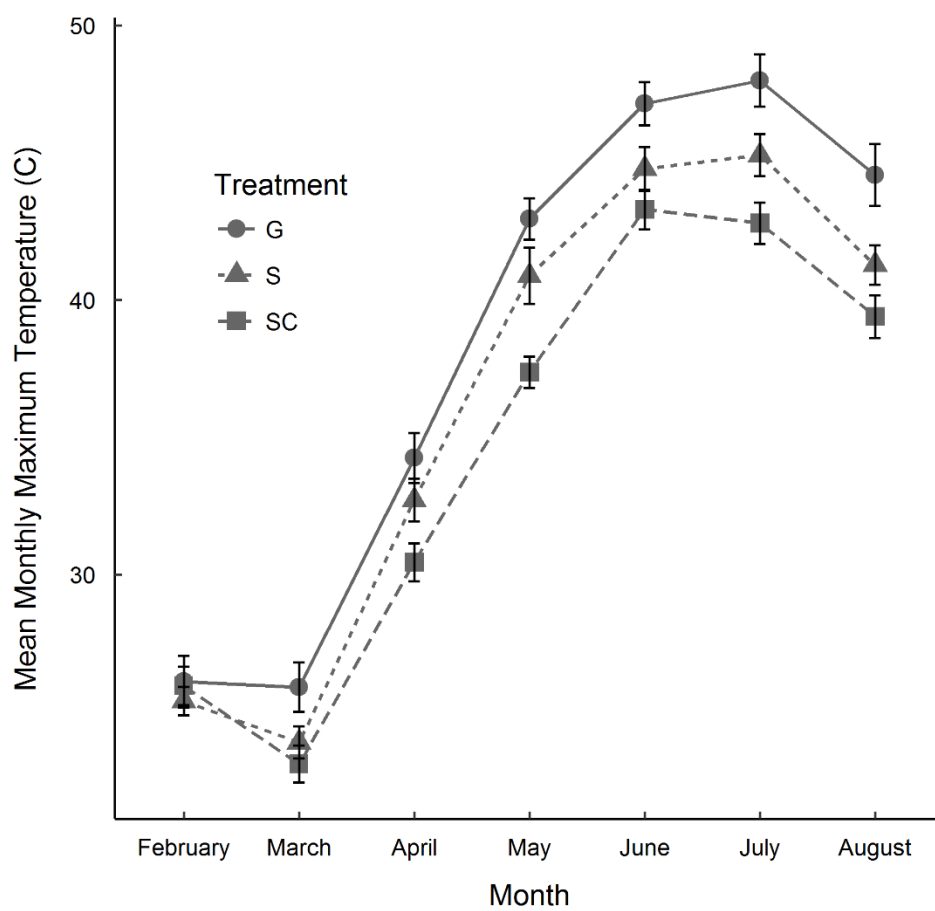


Figure 3. Mean monthly maximum temperature (\pm SE) for each treatment, 5 cm above the ground. All treatments were significantly different.

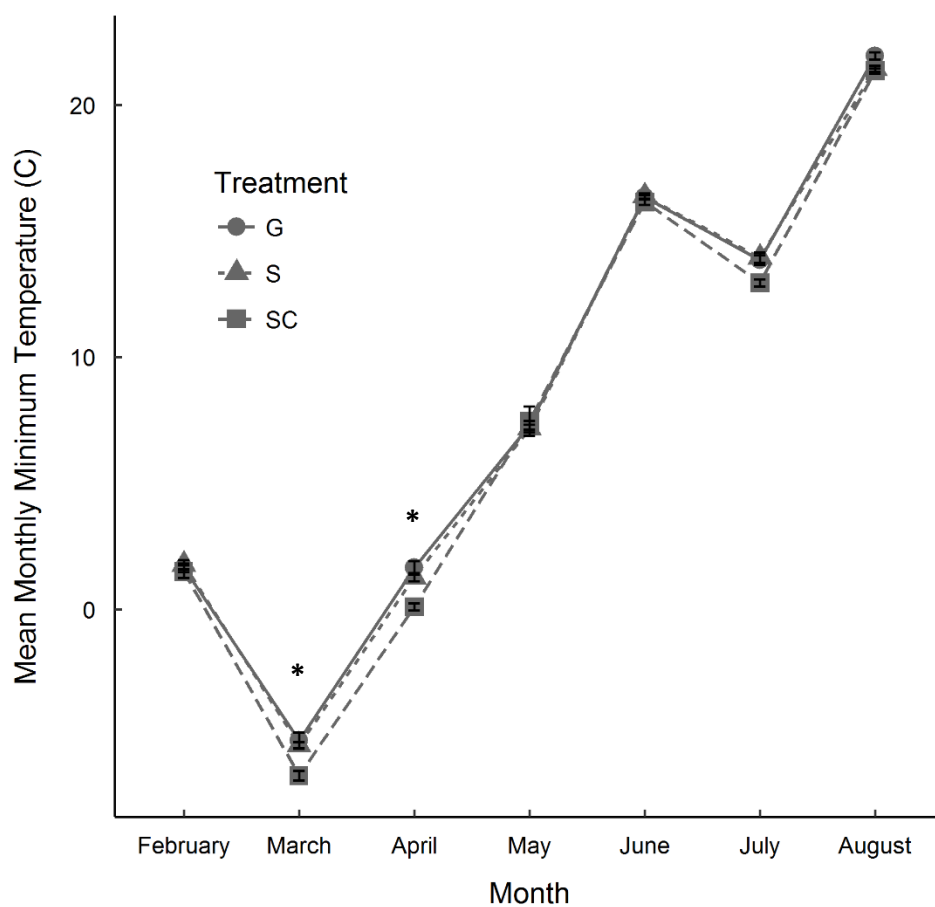


Figure 4. Mean monthly minimum temperature (\pm SE) for each treatment, 5 cm above the ground. Asterisks show months where SC was significantly less than other treatments. In the coldest month, March, SC had the lowest minimum temperature.

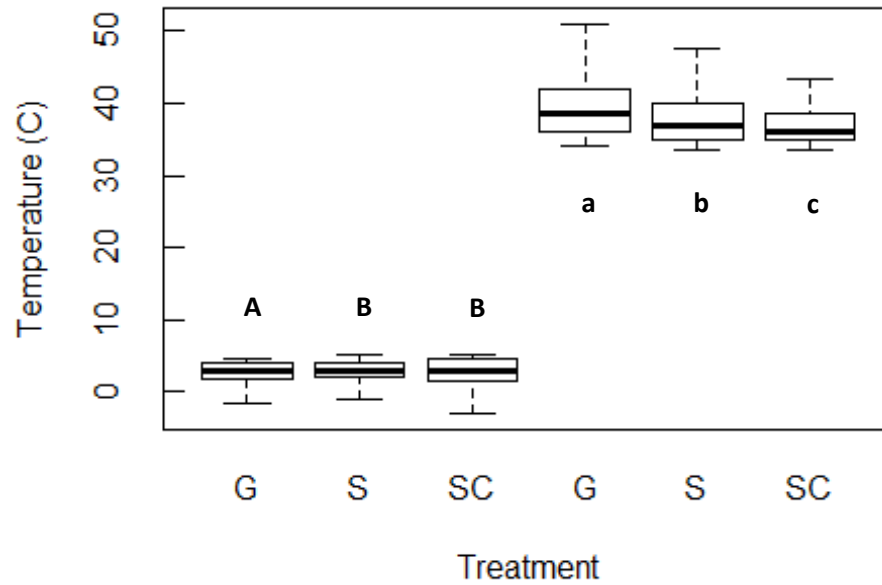


Figure 5. Median (bold line) of 10% hottest and 10% coldest records and quartiles (box and whiskers) of each treatment. Capital letters show significant differences in 10% coldest records, and lowercase letters show differences in 10% hottest records, as determined by Nemenyi tests.

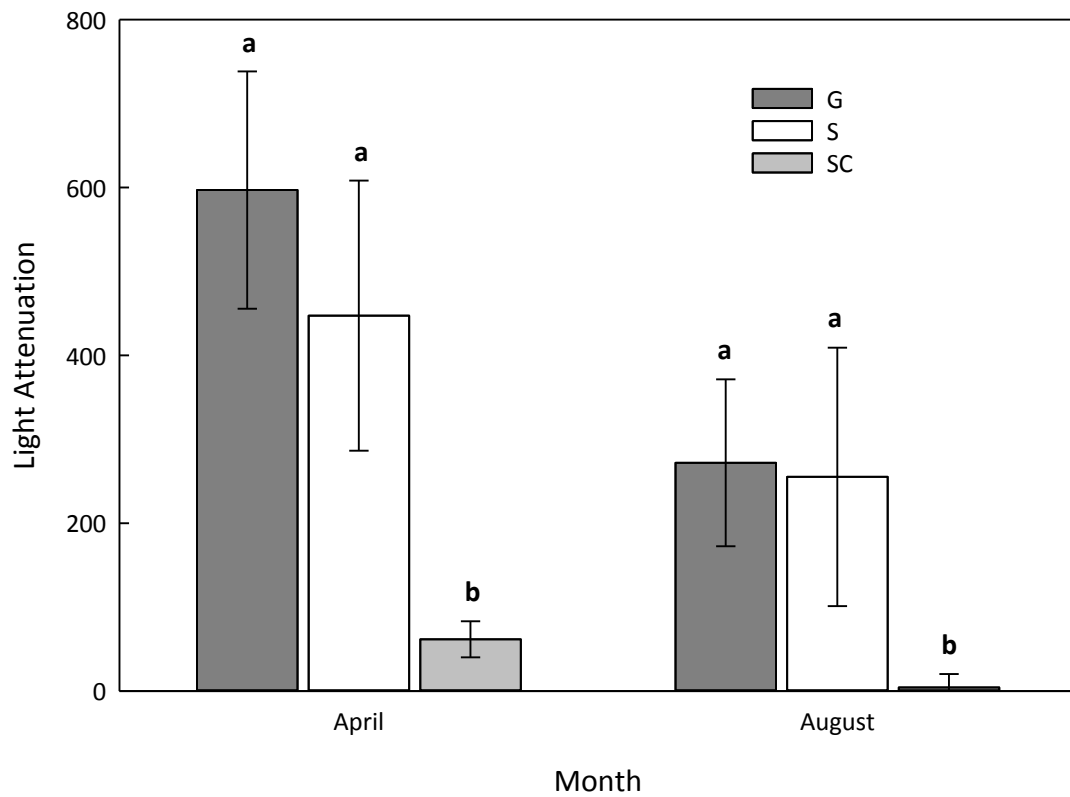


Figure 6. Mean light attenuation (\pm SE) in April and August. Significance letters show differences among treatments. Light attenuation was lowest in SC plots both sampling periods.

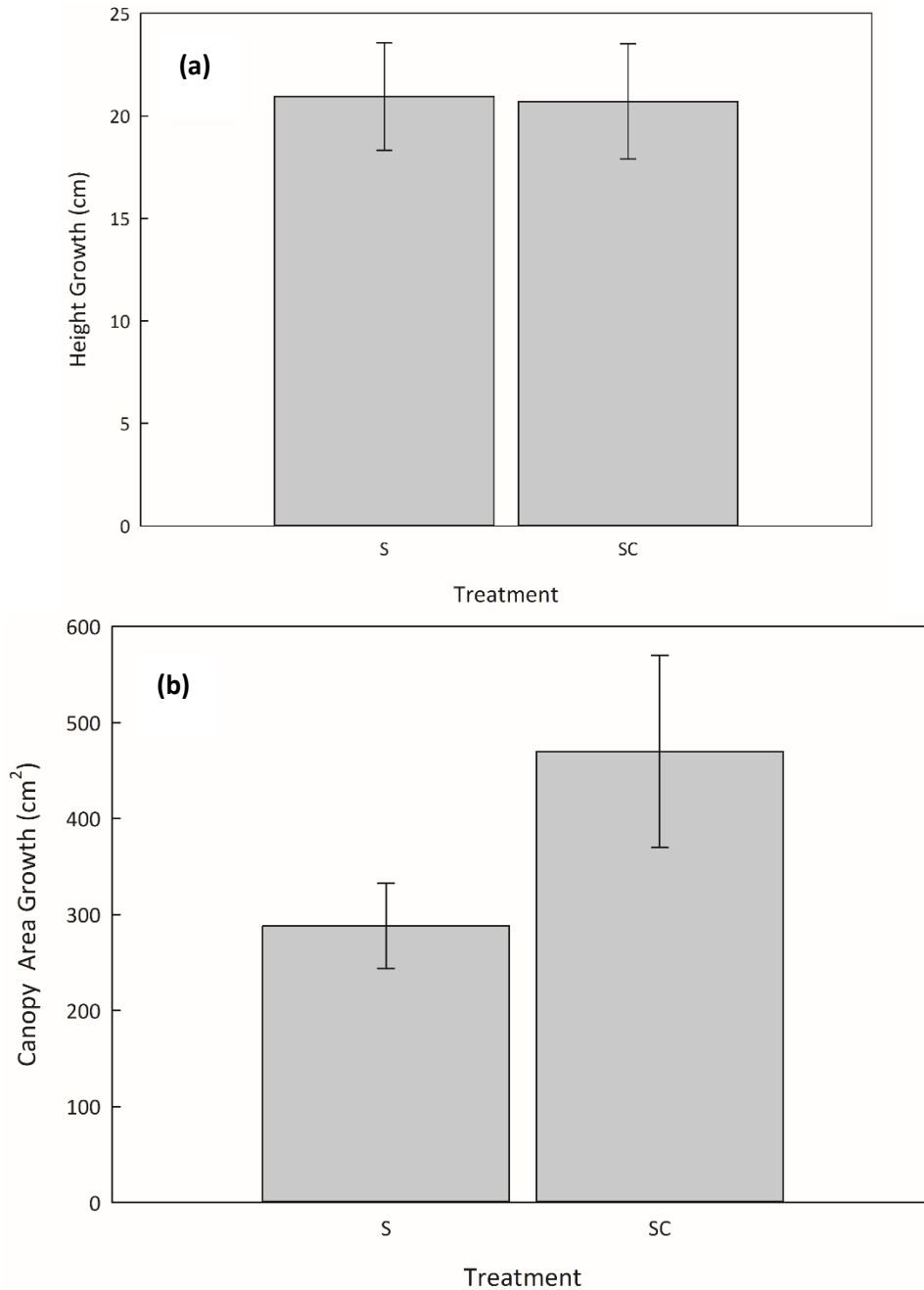


Figure 7. Mean total growth in canopy area (a) and height (b) (\pm SE) from March until August. Change in height or canopy area did not differ, but SC seedlings grew about twice as much in canopy area as S seedlings.

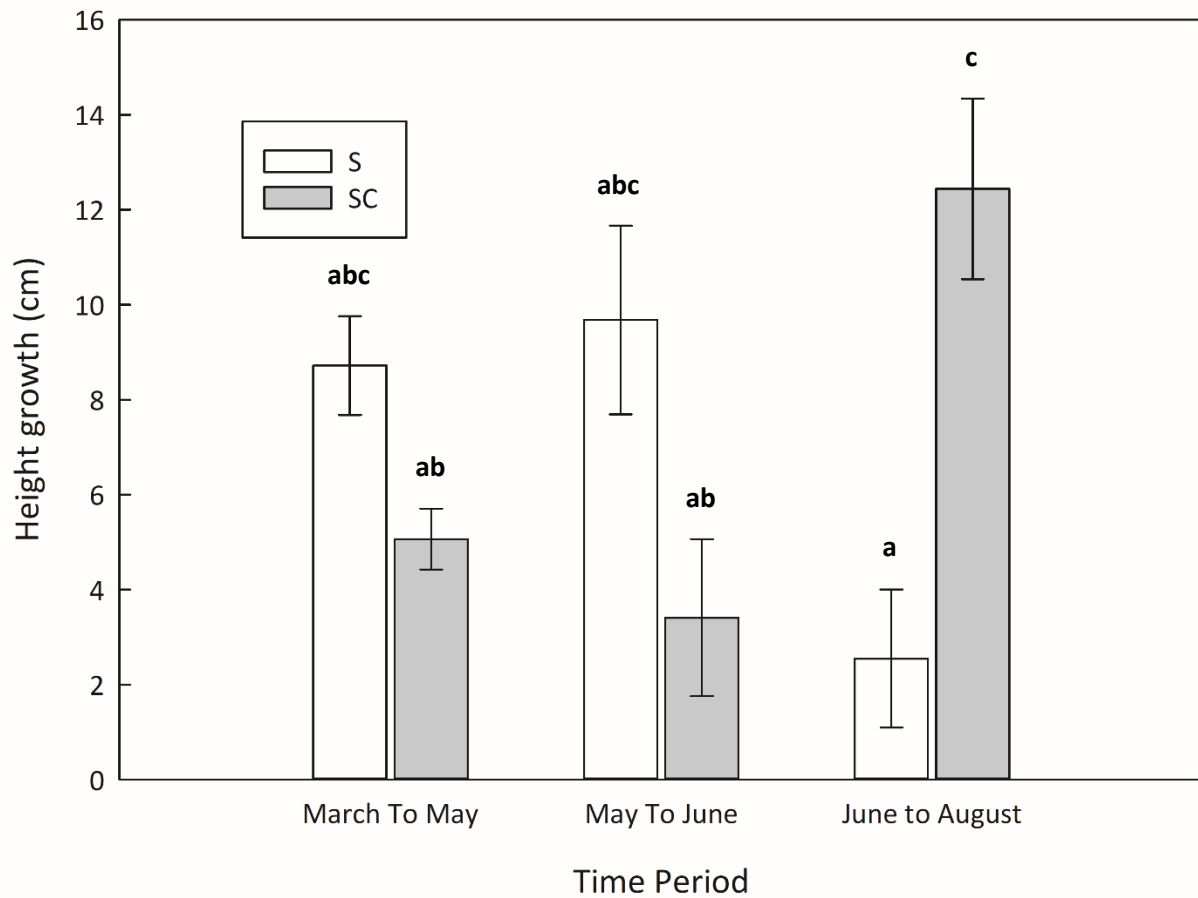


Figure 8. Mean change in height (\pm SE) over two- to three-month intervals shows no difference between treatments until after June. Letters denote significant differences among groups.

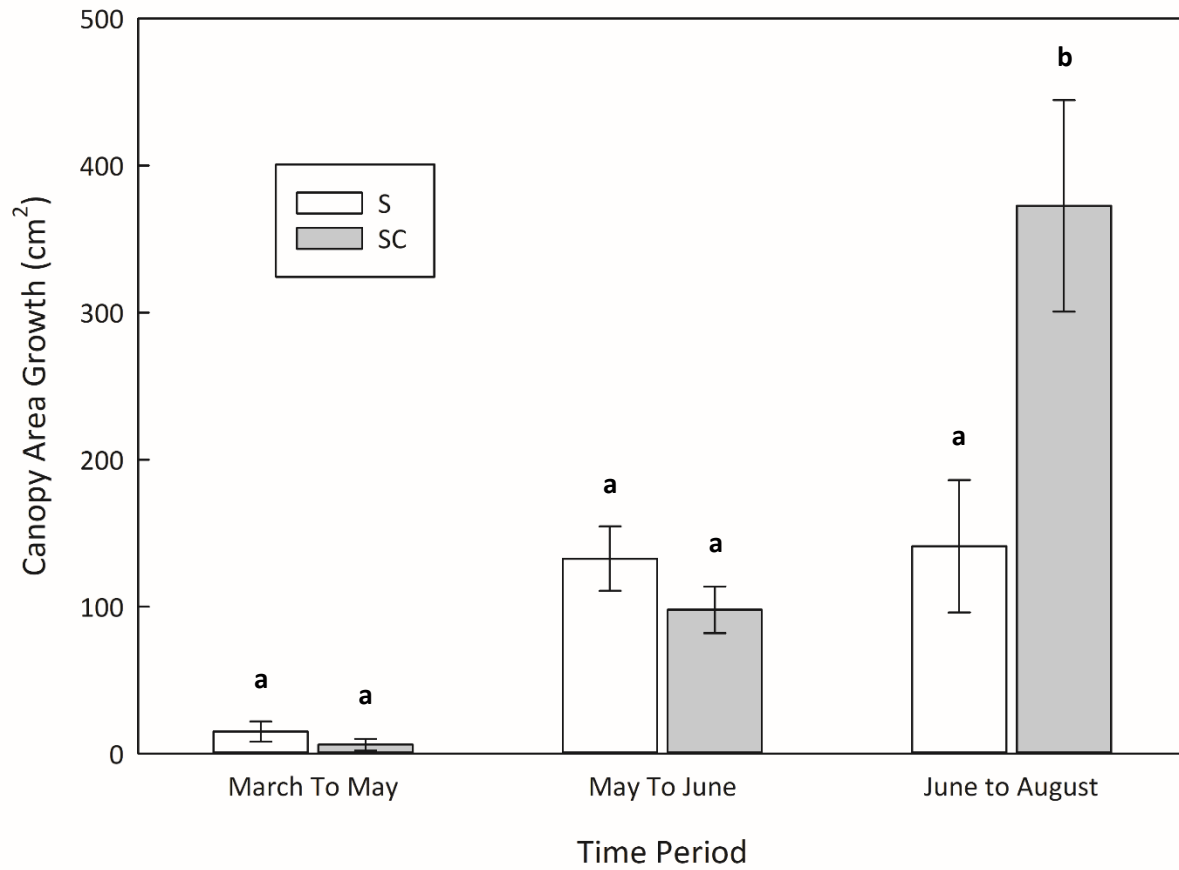


Figure 9. Mean change in canopy area (\pm SE) over two- to three-month intervals shows that SC had greater growth of canopy area during the summer than S. Letters denote significant differences among groups.

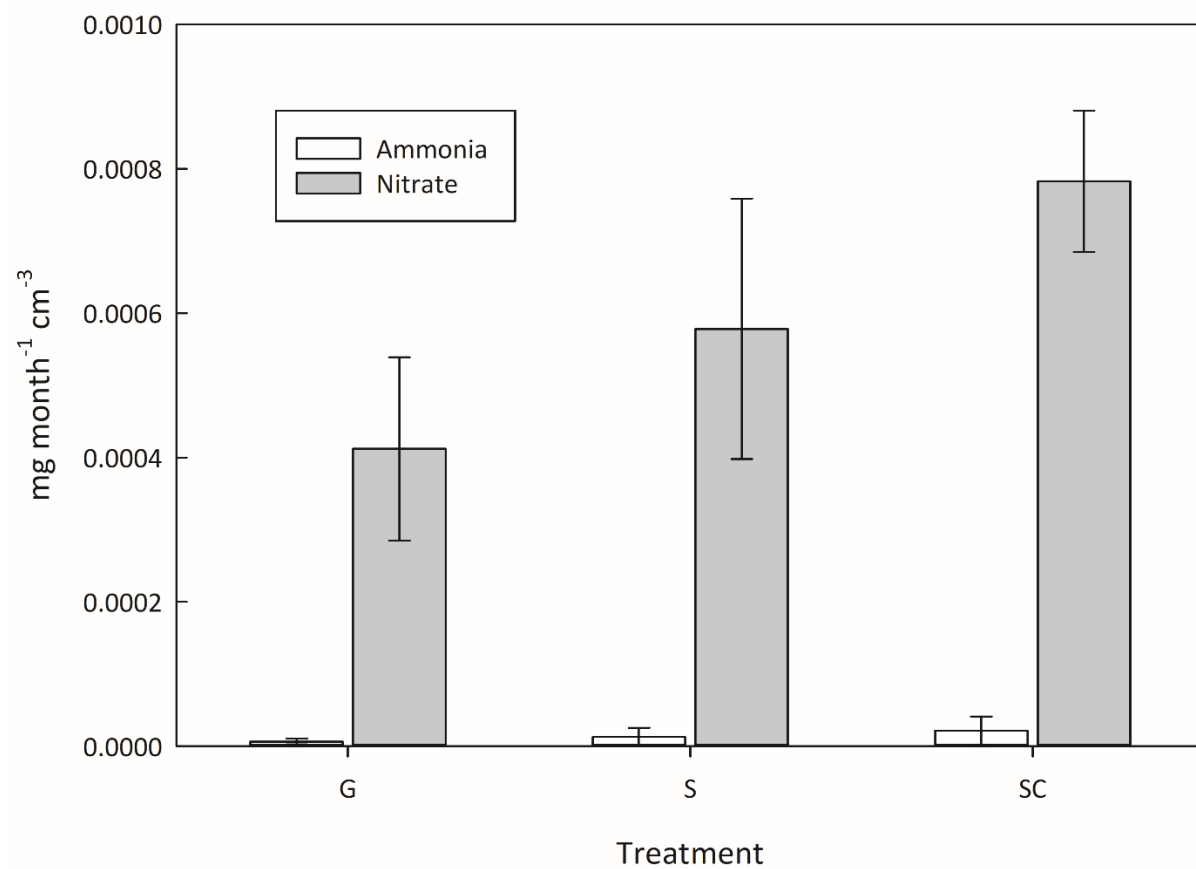


Figure 10. Mean ammonium and mean nitrate (\pm SE) mineralization rates in soils across treatments.

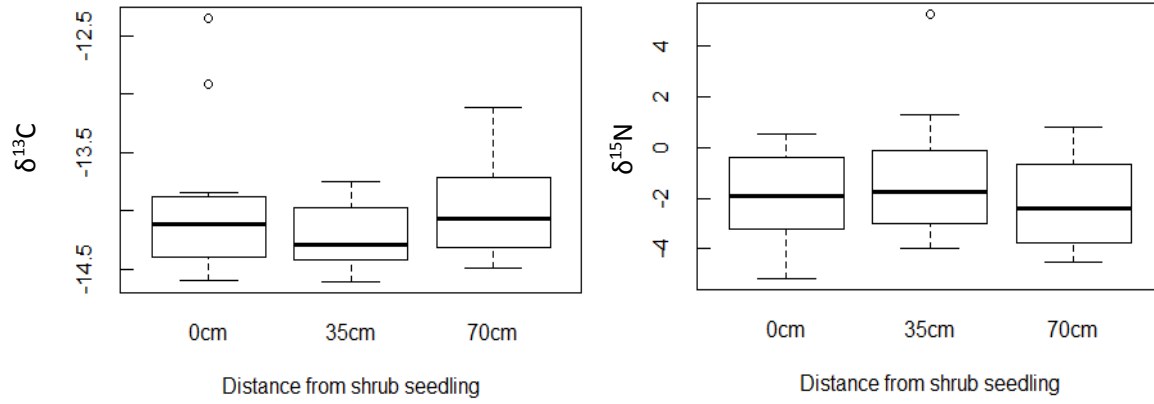


Figure 11. Median $\delta^{13}\text{C}$ (left) and median $\delta^{15}\text{N}$ (right) and quartiles (box and whiskers) of grasses at three distances from shrub seedling. Values in grass tissues did not vary regarding distance from a shrub seedling.

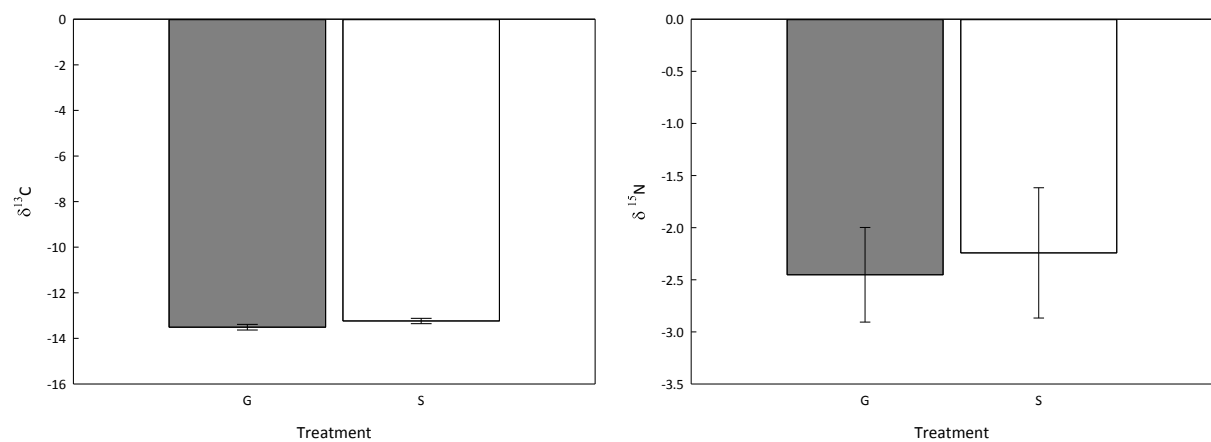


Figure 12. Mean $\delta^{13}\text{C}$ (\pm SE) (left) and mean $\delta^{15}\text{N}$ (\pm SE) (right) of grasses with (S) and without (G) shrub seedling. Values in grass tissues did not differ in the presence or absence of a shrub seedling.



Figure 13. Shrub recovery post Hurricane Michael: (a) nearly all seedlings were leafless after exposure to salt spray in early October 2018, (b) by late October 2018, 6 of 22 re-leafed, (c) by March 2019, only one seedling still had leaves, and (d) only 3 seedlings had living aboveground tissues.

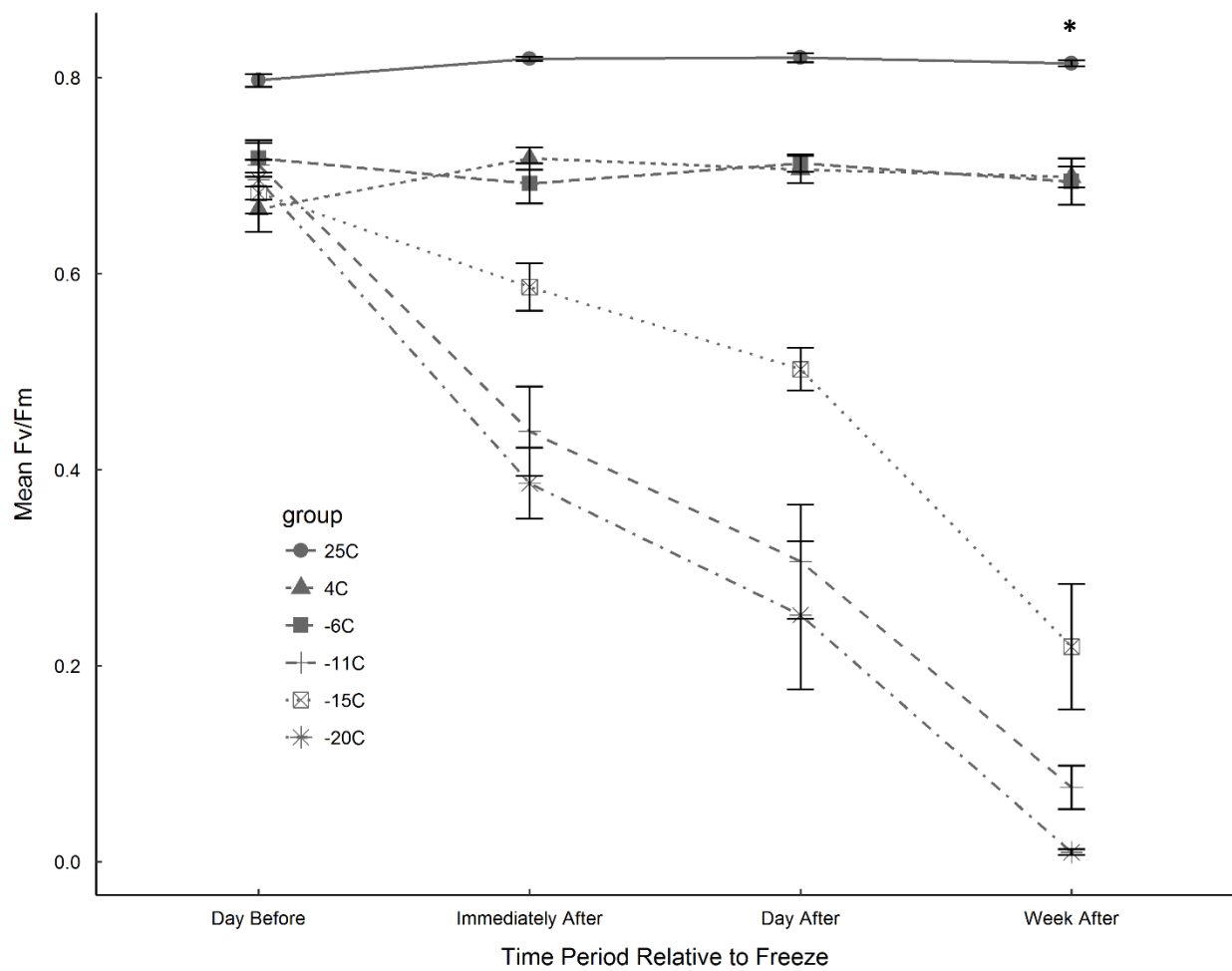


Figure 14. Mean (\pm SE) Fv/Fm for each freeze treatment at each sampling time. Asterisk denotes significant reduction in freeze treatments below threshold ($\leq -11^{\circ}\text{C}$).

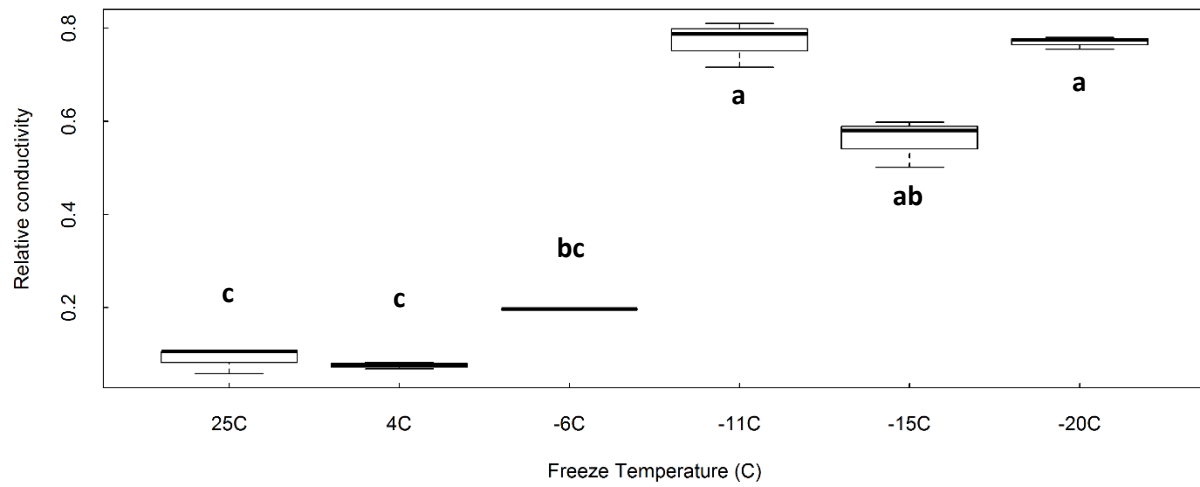


Figure 15. Median relative conductivity (bold line) with quartiles (box and whiskers) across freeze treatments. Letters denote significant differences among treatments.

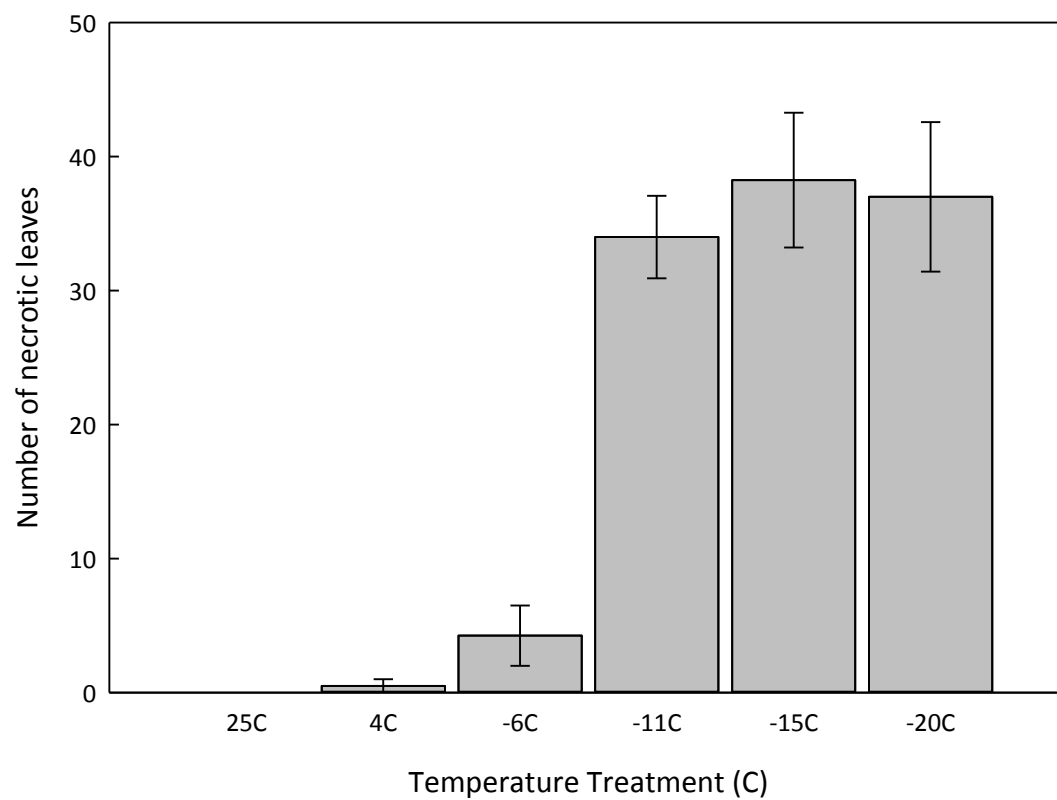


Figure 16. Number of necrotic leaves one week after freezing. Very few leaves became necrotic until one week after freezing.

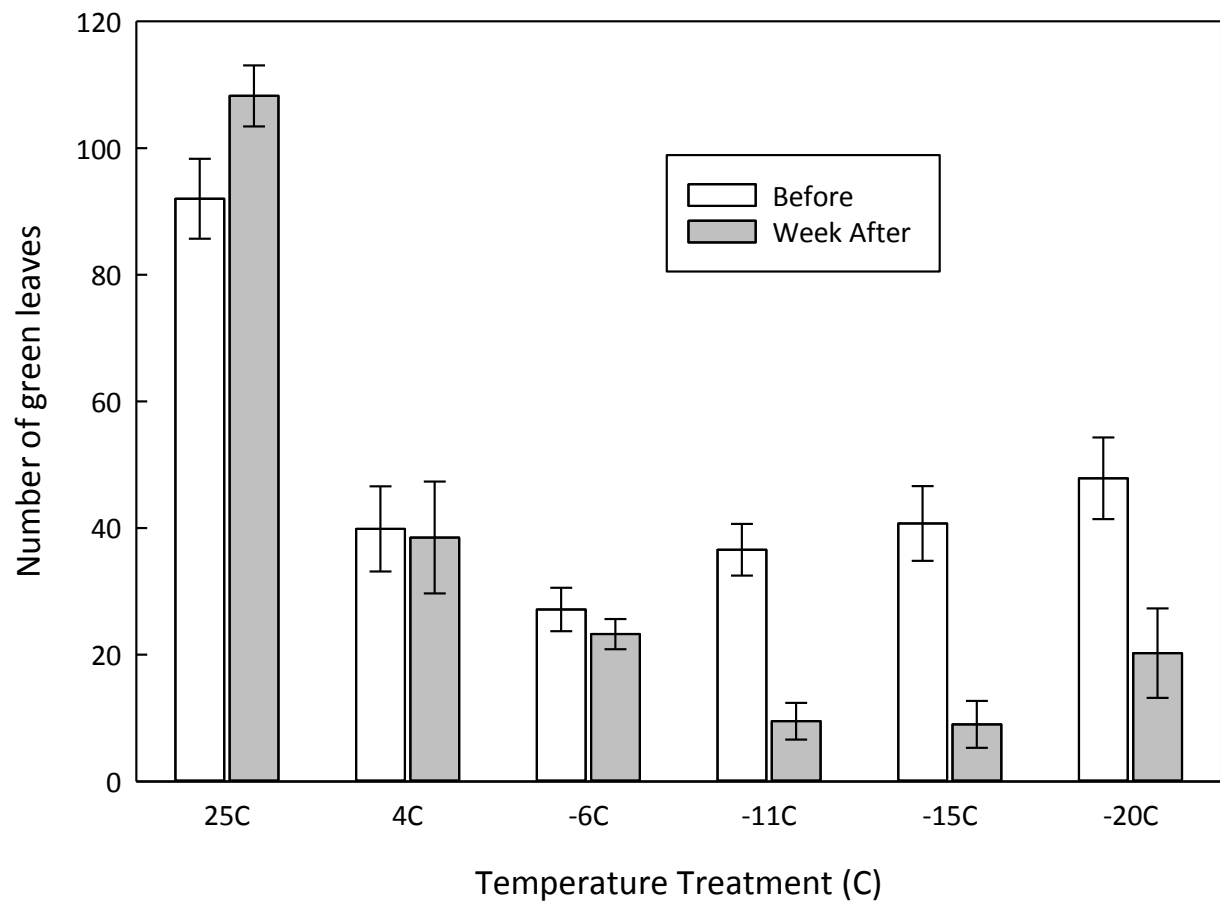


Figure 17. Number of green leaves before and one week after freezing.

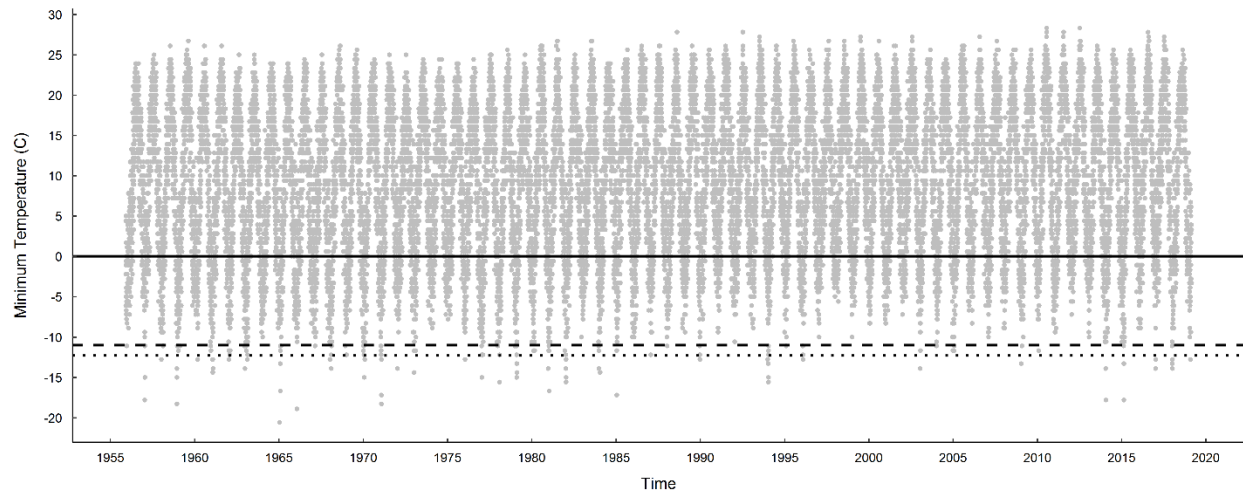


Figure 18. Daily minimum temperatures from 1955 to 2019. Dashed line shows -11°C threshold found in freezing experiment; temperatures below this occurred 44 times since 1985. Dotted line shows -12.3°C (accounting for the 1.3°C insulation provided by grasses); temperatures below this have occurred 20 times since 1985.

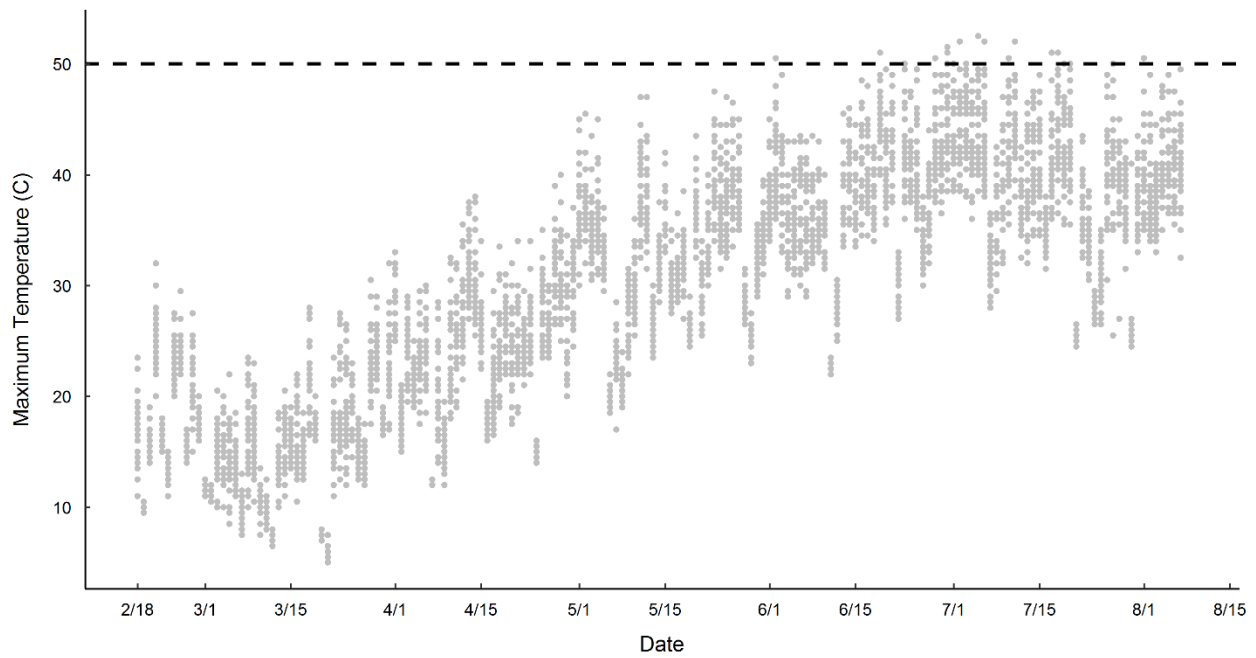


Figure 19. Daily maximum temperatures for each plot from February 18 to August 7, 2019. The dashed line shows 50°C, up to which *M. cerifera* can maintain photosynthetic rates, although reduced. Temperatures >50°C only occurred 13 times during the study only in grass plots.

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Vita

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